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Evidence That Marek's Disease Virus Exists in a Latent State in a Sustainable Fibroblast Cell Line

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Previously, we reported the development of two fibroblastic cell lines (MDV OU2.1 and OU2.2) infected with Marek's disease virus (MDV). The two cell lines, in nonconfluent continuous cultures, displayed characteristics consistent with MDV existing in a latent state. However, presence of distinct plaques in confluent cell monolayers and the ability to transfer cytolytic infection to susceptible birds and primary chick embryo fibroblasts, suggest that, if latent, the virus is easily reactivated from MDV OU2 cell lines. In this report, we present evidence which supports the hypothesis that MDV genomes in MDV OU2 cells are latent. PCR analyses and *in vivo* experiments demonstrate that CHCC-OU2 cells stabilize MDV so that serial *in vitro* passage does not attenuate the virus. Following two years of active culture, MDV genomes in MDV OU2 cells are still oncogenic, similar to that seen in MDV-lymphoblastoid cell lines. Expansion of the 132-bp repeat within MDV *Bam*HI fragments H and D, typical of highly passaged serotype 1 MDV, has not been observed beyond two copies in MDV OU2 cells. Indirect immunofluorescence assays clearly demonstrate that MDV OU2 cells do not express glycoproteins B and I when subconfluent. However, upon reaching confluence these proteins are expressed in readily detectable amounts. Using RT-PCR we demonstrate that glycoproteins E and D are highly expressed in confluent MDV OU2 cells but absent from subconfluent cells, and MDV latency-associated transcripts (LATs), which are antisense to ICP4 transcripts and have been associated with latent MDV infection, are expressed in subconfluent MDV OU2 cells. Coincident with an increase in ICP4 expression, MDV LAT expression is down regulated when MDV OU2 cells become confluent.

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by Marek's disease virus (MDV). MDV is an avian herpesvirus, which is highly infectious and cell associated (Calnek and Witter, 1991). In infected birds, a lytic cycle of MDV replication takes place in differentiating epithelial cells and bursal lymphocytes. Infectious viruses are produced only in feather follicle epithelium and are shed with feather dander and dust (Witter *et al.,* 1972; Calnek *et al.,* 1970). Following an initial burst of lytic infection, T-lymphocytes become latently infected with MDV. Subsequently, chickens infected with virulent, oncogenic (serotype 1) strains of MDV develop aggressive T-cell lymphomas (Calnek and Witter, 1991). Evidence accumulated to date suggests that latent infection of T-lymphocytes is a prerequisite to malignant transformation and neoplastic disease (Shek *et al.,* 1983).

Although MDV cannot produce fully infectious particles (enveloped virus) in tissue culture, cytopathic infections can be produced in monolayers of primary or secondary chicken and duck embryo fibroblasts (CEF and DEF, respectively) as well as chick kidney cells (CKC). CEF, CKC, and DEF support the lytic cycle of MDV *in vitro* (Schat *et al.,* 1989). In addition, more than 90 lymphoblastoid cell lines have been isolated and established from MD tumors (Schat *et al.,* 1991; Akiyama and Kato, 1974; Powell *et al.,* 1974; Calnek *et al.,* 1978; Payne *et al.,* 1981; Nazarian and Witter, 1975). MDV-transformed lymphoblastoid cells are immortalized cell lines which are latently infected with MDV and usually capable of transferring MDV to CEF or DEF *in vitro* and to susceptible chickens *in vivo.*

In MDV lymphoblastoid cell lines a limited set of viral genes have been found to be transcribed (Maray *et al.,* 1988; Sugaya *et al.,* 1990). Transcriptional activity is limited to approximately 20% of the MDV genome, primarily in repeat regions and adjacent sequences. Most transcripts found in MDV lymphoblastoid cells are derived from immediate early (IE) genes (Silver *et al.,* 1979; Schat *et al.,* 1989), suggesting that IE genes could have a significant role in maintenance of latency. Latency-associated transcripts (LATs) which map antisense to IE genes have been described for many herpesviruses. Transcripts antisense to ICP4 have been reported for pseudorabies virus and have been postulated to be involved in latency (Priola *et al.,* 1990). In the case of herpes simplex virus, LATs map antisense to the ICP0 gene (Feldman, 1991).

Epstein–Barr virus (EBV) latently infects human B-lymphocytes, causing both benign and malignant lymphoproliferations. Viral gene expression during latent infection is limited to 11 viral genes, 9 of which are translated. Six of these proteins are nuclear (Ep-

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stein–Barr nuclear antigen (EBNA)-1, -2, -3A, -3B, -3C, -LP), and three latent membrane proteins (LMP)-1, -2A, -2B) (Lee, 1994). Reactivation of EBV replication is induced by ZEBRA (an immediate-early activator encoded by BZLF1 gene) (Countryman and Miller, 1985). BZLF1 is a transcription factor that can activate other viral genes and also affect cellular genes (Flemington and Speck, 1990). In MDV, LATs which map antisense to ICP4 have been detected (Li *et al.,* 1994; Cantello *et al.,* 1994; Mckie *et al.,* 1995). These LAT transcripts appear to be expressed at higher levels in lymphoblastoid cell lines (MSB-1 and RPL1) and are down regulated in lytically infected cells (Li *et al.,* 1994). Although it is possible that MDV LATs play a role in maintenance of latency, definitive evidence is still lacking.

MDV can be rescued from some lymphoblastoid cell lines by cocultivation with primary or secondary CEF and DEF (Schat *et al.,* 1989). In addition, some lymphoblastoid cell lines will induce MD upon injection into susceptible birds (Akiyama *et al.,* 1973, Nazarian *et al.,* 1977). Reactivation of cytolytic infection, however, varies with each cell line.

Serial *in vitro* passage of virulent oncogenic MDV results in loss of MDV tumorigenicity (Churchill and Chubb, 1969). Attenuation of MDV was strongly correlated with an expansion in two regions (*Bam*HI-H and -D), present in MDV long terminal repeat (TR_L) and long inverted repeat (IR_L) regions, respectively (Silva and Witter, 1985; Fukuchi *et al.,* 1985; Chen and Velicer, 1991). It was latter discovered that expansion was due to amplification of a 132-bp direct repeat (DR) sequence found within MDV *Bam*HI-H and -D fragments (Maotani *et al.,* 1986). Tumor induction studies in susceptible birds suggest that cloned virus populations which exhibit an amplification of the 132-bp repeat region have decreased tumorigenic capability. In contrast, viruses which do not contain amplified *Bam*HI-H or -D regions efficiently induce tumors in chickens (Fukuchi *et al.,* 1985).

Recently we reported development of fibroblastic cell lines (MDV OU2.1 and OU2.2) infected with serotype 1 MDV, strain Md11 (Abujoub and Coussens, 1995). MDV OU2 cell lines are similar to certain lymphoblastoid cell lines that MDV infection can be transferred to primary and secondary CEF monolayer culture and MD induced in susceptible birds (Abujoub and Coussens, 1995). However, MDV OU2 cell lines, unlike lymphoblastoid cell lines, are not transformed.

Latency in many viruses results from lack of host factors critical for the expression of viral IE gene products (Garcia-Blanco and Cullen, 1991). Reactivation of these viruses from the latent state is not completely understood, but could be due to activation of specific cellular factors in response to an external stimuli. Stimulation will activate viral regulatory proteins and lead to a state of lytic infection. As a first step in examining the status of MDV in the MDV OU2 cell lines, we report here that MDV exists in a continuous latent state in MDV OU2 cell lines and virus lytic cycle is activated upon confluency. Virus produced from MDV OU2 cell lines remained virulent and oncogenic after more than 30 *in vitro* passages. In addition, continuous cultivation in CHCC-OU2 cells appears to stabilize MDV genomes. For approximately 2 years of continuous culture, only a minor expansion of an unstable 132-bp DR sequence (het region) was detected in MDV OU2 cells. Our results suggest the MDV OU2 cell system may represent an ideal *in vitro* system to study factors involved in the switch between lytic and latent herpesvirus infections. In addition, stabilization of MDV genes will have important applications in production of recombinant and mutant MDV.

MATERIALS AND METHODS

Cells and virus

Preparation, propagation, and infection of CEF cells with MDV were performed as described previously (Glaubiger *et al.,* 1983; Coussens and Velicer, 1988). The very virulent MDV strain Md11 was used at cell culture passage levels 15, 28, 35, 48, and 86 (Md11p15, Md11p28, Md11p35, Md11p48, and Md11p86, respectively). CHCC-OU2 (Ogura and Fujiwara, 1987), MDV OU2.2, and MDV OU2.1 cells were maintained as described previously (Abujoub and Coussens, 1995). MDV OU2 cells were used at cell culture passages 8, 12, 16, 23, and 31 (MDV OU2.2p8 , MDV OU2.2p12, MDV OU2.2p16, MDV OU2.2p23, and MDV OU2.2p31) for PCR, and RT-PCR analyses . MDV OU2.1 and OU2.2 cells are passaged every 10–15 days prior to formation of confluent monolayers.

Preparation of cellular DNA and polymerase chain reaction (PCR)

Total cellular DNA was extracted from CEF, CEF/Md11, MSB-1, CHCC-OU2, and MDV OU2 cells by standard methods (Sambrook *et al.,* 1989). Total cellular DNA was used as a template for PCR amplification of the 132-bp DR sequences. The upstream primer (5′-TGCGATGAA-AGTGCTATGGAGG-3′) anneals 3-bp 5′ to the 132-bp DR sequences, while the downstream primer (5′-GAGAAT-CCCTATGAGAAAGCGC-3′) anneals 6 bp from the 3′ end of the 132-bp DR sequences. PCR using these two primers amplifies a 317-bp fragment when two copies of the DR sequence are present (Silva, 1992). PCR conditions were slightly modified from those suggested by Silva (1992). Briefly, 200 ng of total cellular DNA was mixed with 20 m*M* of each dNTP , 20 μM of each oligonucleotide primer pair, 10 μl 10$\times$ PCR reaction buffer (Life Technologies, Gaithersburg, MD), 1.5 m*M* $MgCl_2$, and 1.0 U *Taq* polymerase (Life Technologies). PCR reactions were performed using a GeneAmp 9600 thermal cycler (Perkin–Elmer–Cetus, Norwalk, CT). Following an initial denatur-

ing step at 95° for 5 min, DNA was amplified during 25 cycles of 95° for 30 sec, 67° for 30 sec, and 72° for 30 sec. PCR reactions were completed by a final elongation step at 72° for 10 min. A negative control with CHCC-OU2 DNA was included in each PCR reaction. Amplification of an 850-bp fragment of the pp38 gene was performed as previously described (Abujoub and Coussens, 1995). PCR products were analyzed on 6% polyacrylamide gels, stained with ethidium bromide, and photographed under ultraviolet light. Sizes of amplified fragments were determined by comparison to a 1-kb DNA ladder marker (Life Technologies).

RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from CHCC-OU2, MDV OU2.2p31, Md11p35, and MSB-1 cells using the Trizole reagent (Life Technologies) according to the manufacturer's recommendation. Prior to use, RNA was treated with 10 units RNase-free RQ1 DNAase (Promega, Madison, WI) for 30 min at 37°. Coupled cDNA synthesis and PCR amplification of extracted RNAs was carried out in two steps. First, cDNA synthesis was performed in 250 m*M* Tris–HCl (pH 8.3), 375 m*M* KCl, 15 m*M* $MgCl_2$, 100 m*M* dithiothreitol, and 10 m*M* each dATP, dCTP, dGTP, and dTTP. First strand synthesis mixture also contained 0.5 U RNasin (Promega), 200 U Superscript II Reverse Transcriptase (Life Technologies), and 15 μg of total cellular RNA. One micromolar of oligonucleotide corresponding to nt 411–435 (complementary) of M49 cDNA clone of MDV (Li *et al.,* 1994) (5′-CGTCGGACATGTTTCCAGATC-GCC-3′) was used as a downstream primer in order to amplify MDV LATs transcripts, whereas 1 μM of oligonucleotide corresponding to nt 98–122 of M49 cDNA clone of MDV (Li *et al.,* 1994) (5′-CGTTGGACGGCTCGGCGG-ACTTGGG-3′) was used as a downstream primer to amplify ICP4 transcripts. Oligonucleotide primers D1 (5′-AGTGAGTCCAGTGTAAACCATCC-3′) and E1 (5′-CAG-AATGTCAATGTTGGATGCG-3′) were used as downstream primers to amplify glycoproteins D and E transcripts, respectively. Reactions were incubated at 42° for 60 min, followed by enzyme inactivation for 15 min at 70°. Second, the cDNA synthesized in the first step was used as a template for PCR reaction as follows: 10% of the first strand reaction was amplified in 200 m*M* Tris–HCl (pH 8.4), 500 m*M* KCl , 1.5 m*M* $MgCl_2$, 20 m*M* of each dNTP, and 10 μM each of oligonucleotide corresponding to nt 98–122 of M49 cDNA clone of MDV and nt 411–435 (complementary) (Li *et al.,* 1994) for amplifying MDV LATs and MDV ICP4. PCR reactions were performed using a GeneAmp 2400 thermal cycler (Perkin–Elmer–Cetus). Following an initial denaturing step at 95° for 5 min, DNA was amplified during 30 cycles of 95° for 45 sec, 62° for 45 sec, and 72° for 45 sec. PCR reactions were completed by a final elongation step at 72° for 15 min. A negative control without template and a positive control with Md11p35 DNA was included in each RT-PCR reaction. PCR conditions for glycoproteins D and E were similar to those of MDV ICP4 and LATs, except for using 10 μM of oligonucleotide primers D1 and D2 (5′-TACGTGAATATGCCAACTGC-3′) as an upstream primer and oligonucleotide primers E1 and E2 (5′-GAA-TCGCTAAGTCTGAATGG-3′) as an upstream primer, respectively. Also, the annealing temperature has been changed to 56° to match the T_m of these oligonucleotide primers.

Inoculation of chickens with cells and virus

In vivo experiments were performed using specific pathogen-free chickens (SPF), obtained from SPAFAS (Chicago, IL). Chicks were divided into three groups of 5 chicks per group at 1 day of age, and groups inoculated intraperitoneally with: (A) 2.0×10^6 uninfected CHCC-OU2 cells, (B) 2000 plaque forming units (PFU) of MDV OU2.2p8 cells, or (C) 2000 PFU of MDV OU2.2p23.

Birds were euthanized and necropsied upon severe signs of morbidity. Blood was collected, and peripheral blood leukocytes (PBLs) were isolated as described (Tardef and McQueen, 1993) and cocultivated with secondary CEF cells as an assay for production of viable virus. Various tissues, including heart, liver, kidney, and spleen were frozen for subsequent DNA isolation. Total tissue-specific (Kidney) DNA was isolated and used as template for PCR amplification of the 132-bp DR sequence and for an 850-bp region of MDV pp38 gene sequences.

Indirect immunofluorescence antibody (IIFA) staining

Uninfected CEF, CEF infected with Md11p15, CHCC-OU2, and MDV OU2.2 cells, grown on glass cover slips in 35-mm tissue culture plates, were used for indirect immunofluorescence labeling according to standard protocols (Harlow and Lane, 1988; Hong *et al.,* 1995). CEF and CHCC-OU2 cells were processed 3 days postplating on cover slips. CEF infected with Md11p15 were processed 1 day after displaying plaques characteristic of MDV infection. MDV OU2.2 were processed either when they were 60–80% confluent (subconfluent) or 5 days after forming a confluent monolayer (confluent). Cells were washed with cold PBS, fixed and permeabilized with 2 ml of ice-cold acetone:methanol (1:1) for 3 min at room temperature with gentle agitation. Following three washes with cold PBS, cells were preincubated with 3% BSA in PBS for 1 hr at room temperature. Anti-MDV/gB (Silva and Lee, 1984) monoclonal antibody (generously provided by Dr. Lucy Lee, USDA-ADOL) was added to the cells at a 1:40 dilution in PBS and incubated for 1 hr at room temperature. Anti-MDV/gI polyclonal antibody (generously provided by Dr. Lee Velicer, Department of Microbiology, Michigan State University) was added to cells in a 1:20 dilution in PBS and incubated for 1 hr

at room temperature. After extensive washing with PBS, sheep anti-mouse IgG (whole molecule) conjugated with fluorescein-5′-isothiocyanate (FITC) (Sigma) was used as secondary antibody for cells incubated with anti-MDV/gB as primary antibody. Goat anti-rabbit IgG (whole molecule) conjugated with FITC (Sigma, St. Louis, MO) was used as a secondary antibody for cells incubated with anti-MDV/gI as a primary antibody. All secondary antibodies were diluted 1:20 in PBS. After extensive washing with PBS, Slow-Fade (Molecular Probes, Eugene, OR) was added prior to mounting to glass slides to minimize quenching of fluorescence. Cells were photographed on an Olympus BH-2 fluorescence microscope.

RESULTS

MDV is stably maintained in subconfluent MDV OU2 cells

Recently we reported development of two fibroblastic cell lines (MDV OU2.2 and OU2.1) capable of supporting replication and growth of MDV (Abujoub and Coussens, 1995). MDV OU2 cells cannot be visibly distinguished from uninfected parental CHCC-OU2 cells when grown at a subconfluent level (Figs. 1A and 1B). Subconfluent MDV OU2.2 cells do not display any signs of lytic MDV infection and exhibit a doubling time only slightly shorter than parental cells. Within 5 days of becoming confluent, monolayers of MDV OU2 cells display plaques similar in appearance to those observed in MDV-infected primary or secondary CEF cells (Figs. 1C and 1D). Thus MDV OU2 cells have been viable in cell culture for almost 2 years and display plaques only when allowed to reach confluence.

Within MDV OU2 cells, MDV may exist in a latent state and is reactivated when cells become confluent. Alternatively, cytolytic infection of CHCC-OU2 cells by MDV may be offset by cellular growth in subconfluent cultures. In this case, plaques become visible only when contact inhibition decreases cellular growth. To distinguish between these possibilities, the nature of MDV infection in MDV OU2 cells was examined.

Detection of viral proteins by IIFA staining

MDV OU2 cells express pp38 and pp14 in quantities sufficient to be detected by Western blot analysis (Abujoub and Coussens, 1995). However, expression of other MDV antigens, particularly those of structural glycoproteins, was not detectable by Western blot analysis (Abujoub and Coussens, 1995). Therefore, we compared MDV antigen expression by IIFA staining of subconfluent and confluent MDV OU2 cells. Anti-MDV/gB (Silva and Lee, 1984) monoclonal antibody IAN 86 (generously provided by Dr. Lucy Lee, USDA-ADOL) detected a protein consistent with MDV gB in confluent MDV OU2.2p16, but not in subconfluent MDV OU2.2p16 cells (Figs. 2D and 2B, respectively). Similarly, anti-MDV/gI (Brunovskis, and Velicer, 1995) polyclonal antibodies detected a protein consistent with MDV gI in confluent MDV OU2.2p16, but not in subconfluent cells (Figs. 3D and 3B, respectively). A similar protein was not detected in either subconfluent or confluent CHCC-OU2 cells (A and C in Figs. 2 and 3). Uninfected CEF and Md11p15/CEF cells were used as negative and positive controls, respectively (data not shown). Together, results of IIFA staining, Western blot analysis (Abujoub and Coussens, 1995), and MDV OU2 cell growth characteristics suggest that fully lytic growth of MDV and corresponding expression of late proteins is initiated only after MDV OU2 cells form a confluent monolayer.

Detection of late structural glycoproteins expression using RT-PCR

RT-PCR was used to compare MDV gene expression between subconfluent and confluent MDV OU2 cells. Oligonucleotide primers specific for glycoproteins E and D amplified 530- and 560-bp fragments, respectively, from confluent MDV OU2 cells but not from subconfluent MDV OU2 cells (Figs. 4A and 4B). A similar pattern of expression was detected in MD11p35 lytically infected CEF cells and no expression was detected in uninfected CHCC-OU2 cells (Figs. 4A and 4B). No expression of gD was detected in MSB-1 cells but very low level of gE expression was detected. This low level of expression may be due to the spontaneous viral reactivation within MSB-1 cells.

MDV LATs are expressed in subconfluent MDV OU2 cells

We used RT-PCR to determine if antisense ICP4 mRNA's could be detected in MDV OU2 cell lines under subconfluent and confluent conditions. Total cellular RNA isolated from CHCC-OU2 cells, MDV-OU2 cells (subconfluent and confluent), MSB-1 cells, and Md11p35/CEF cultures was used for RT-PCR to detect expression of MDV LATs. A 335-bp fragment corresponding to the 5′ end of M49 cDNA which maps antisense to the MDV ICP4 gene (Li *et al.,* 1994) was detected in RNA isolated from subconfluent MDV OU2 cells, MSB-1 cells, and occasionally in confluent MDV OU2 cells, but was not detected in RNA isolated from lytically infected Md11p35/CEF or uninfected CHCC-OU2 cells (Fig. 5A). A fragment of approximately 230 bp amplified during RT-PCR of MSB-1 cells RNA may represent an alternatively spliced variant of MDV LATs (Fig. 5A, lane 6).

To verify that products amplified in RT-PCR reactions were indeed antisense to the MDV ICP4 homolog gene, PCR products were transferred to a Zeta-probe membrane and probed with DNA corresponding to the MDV ICP4 gene. Hybridization of radioactively labeled MDV ICP4 probes to RT-PCR products from reaction with MDV

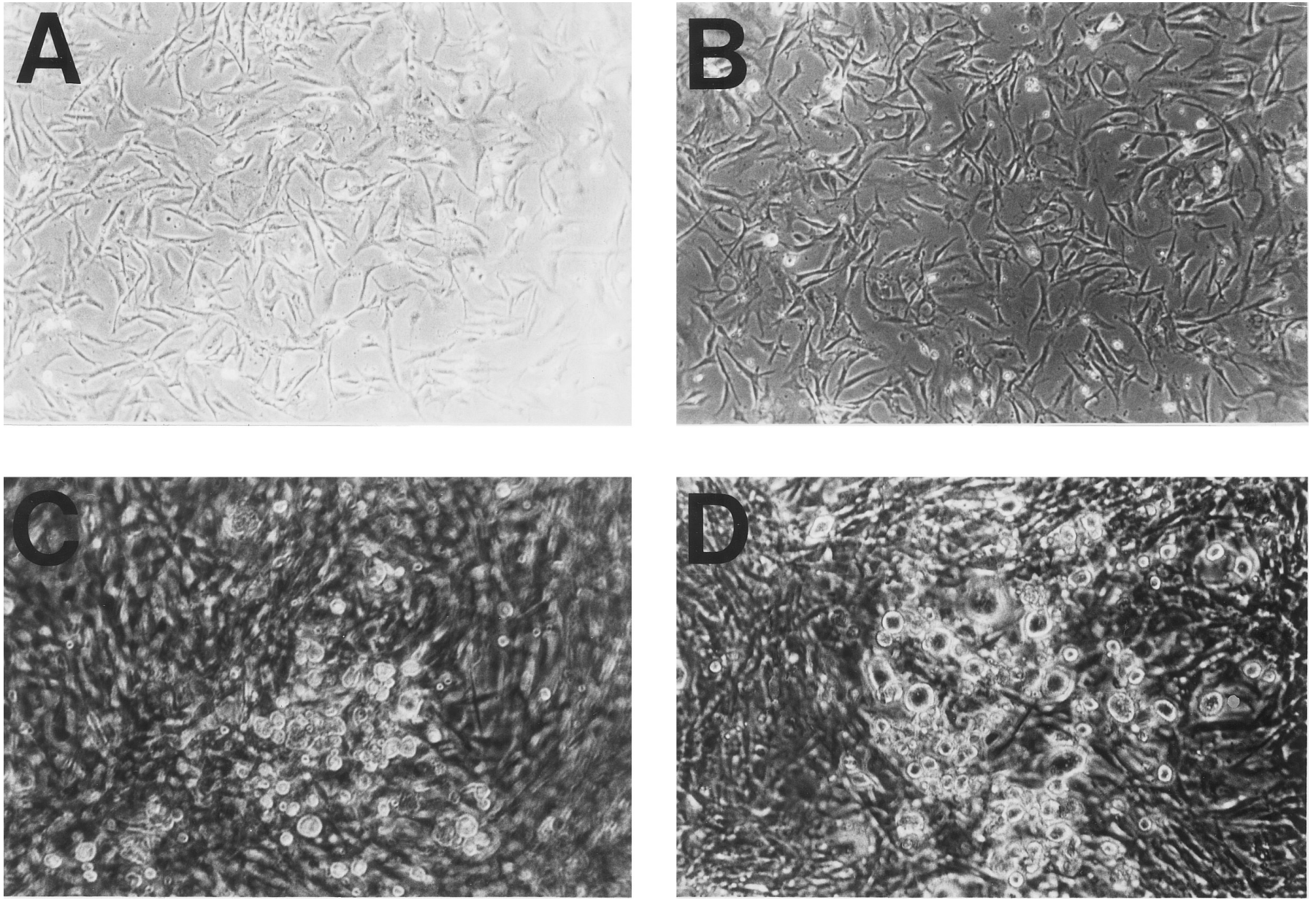

FIG. 1. Subconfluent MDV OU2.2p23 and MDV OU2.1p23 cells display a cobblestone appearance, similar to that seen with uninfected parental CHCC-OU2 cells (A and B, respectively). At 5–7 days after cells became confluent, numerous plaques consistent with MDV lytic infection are observed on cultures of MDV OU2.2p23 and MDV OU2.1p23 cells (C and D, respectively).

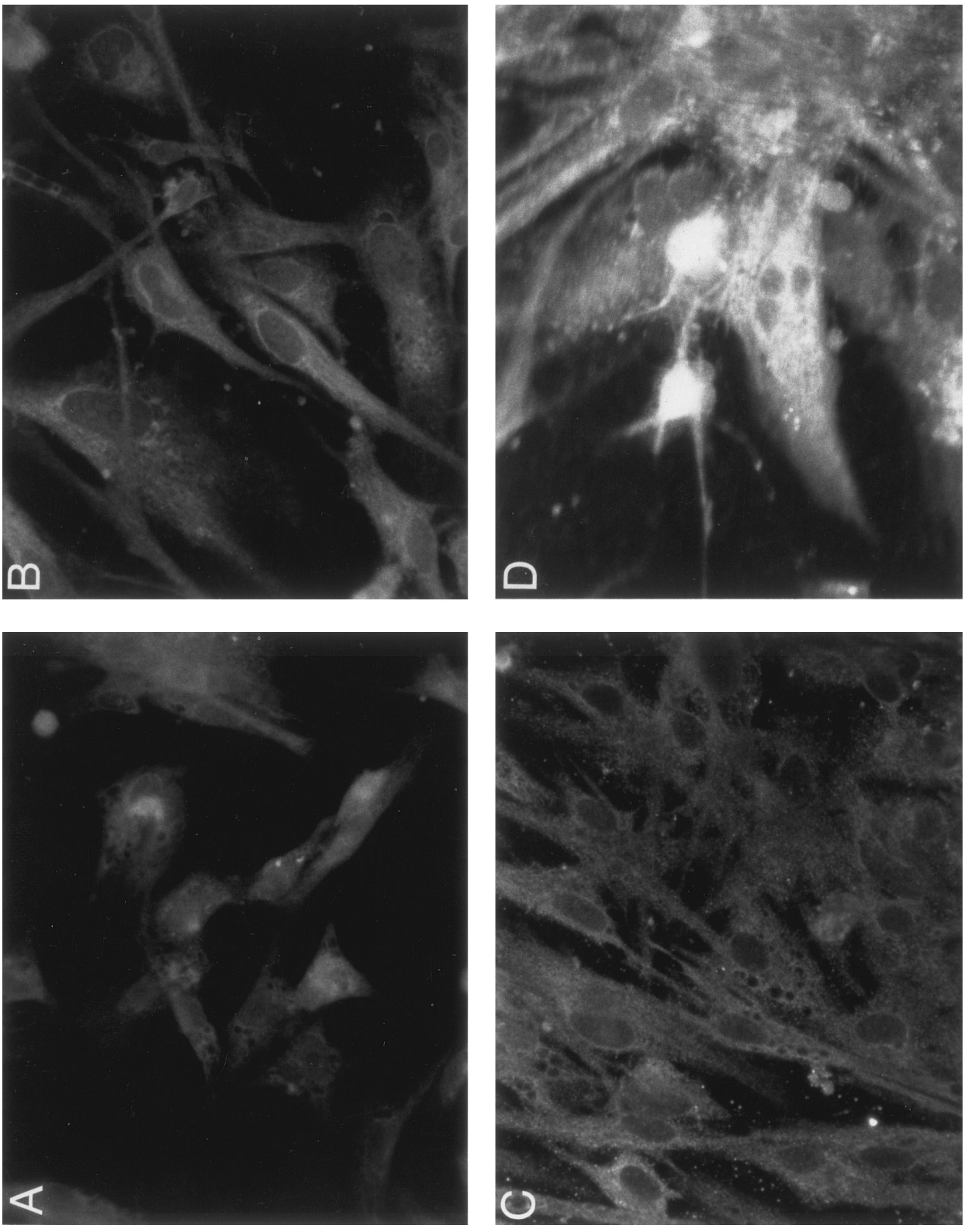

FIG. 2. IIFA staining with monoclonal antibody IAN86 specific for MDV gB homologue. Subconfluent CHCC-OU2, subconfluent MDV OU2.2, confluent CHCC-OU2, and confluent MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described under Materials and Methods. Sheep anti-mouse IgG conjugated with FITC (Sigma) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40× objective and 3.3× photo eyepiece. (A) Subconfluent CHCC-OU2 cells. (B) Subconfluent MDV OU2.2 cells. (C) Confluent CHCC-OU2 cells. (D) Confluent MDV OU2.2 cells.

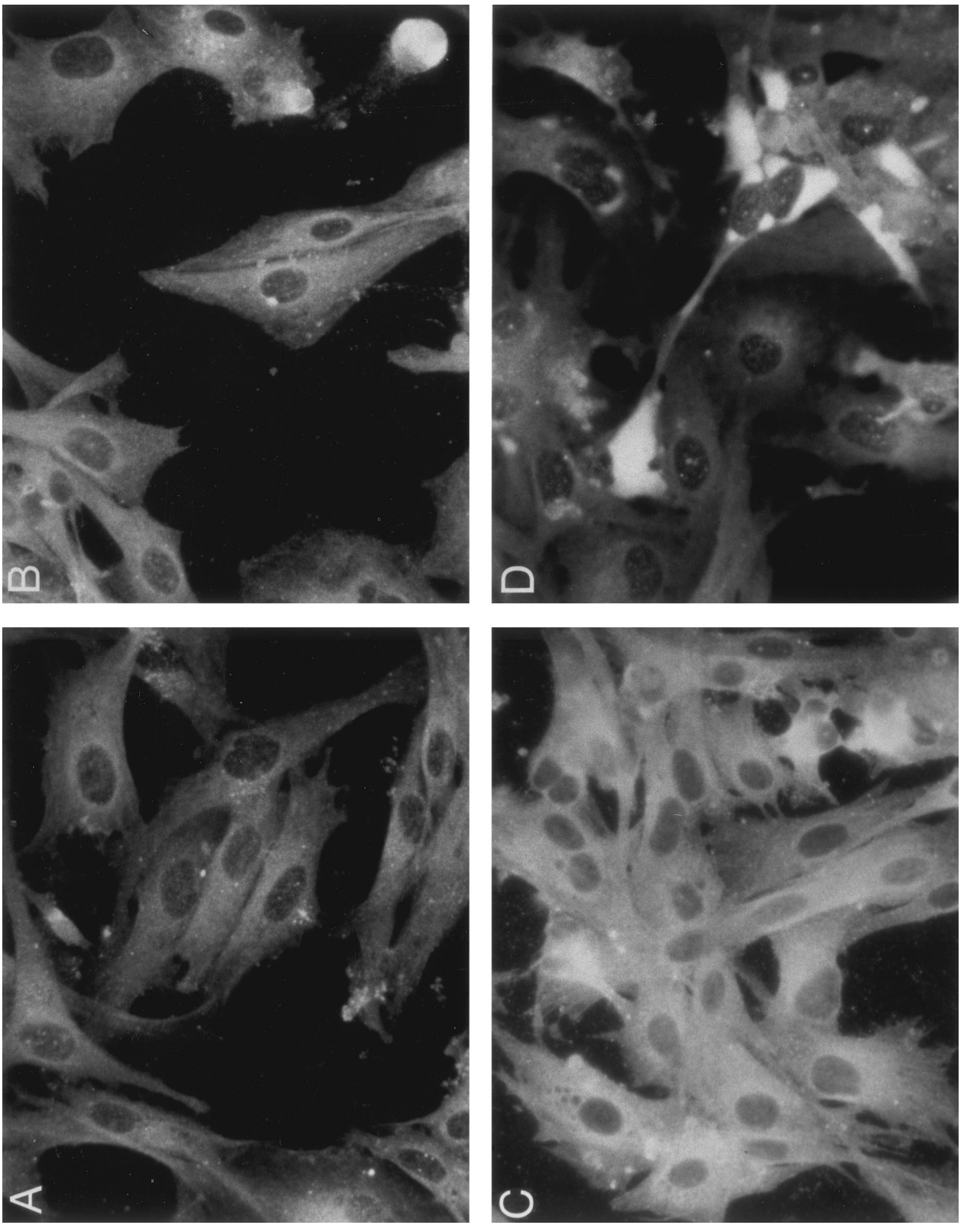

FIG. 3. IIFA staining with polyclonal antisera against MDV gI homologue. Subconfluent CHCC-OU2, subconfluent MDV OU2.2, confluent CHCC-OU2, and confluent MDV OU2.2 cells were grown on glass cover slips. Cells were fixed and stained as described under Materials and Methods. Sheep anti-rabbit IgG conjugated with FITC (Sigma) was used as a secondary antibody. Cells were photographed on an Olympus BH-2 fluorescence microscope with a 40X objective and 3.3X photo eyepiece. (A) Subconfluent CHCC-OU2 cells. (B) Subconfluent MDV OU2.2 cells. (C) Confluent CHCC-OU2 cells. (D) Confluent MDV OU2.2 cells.

OU2 and MSB-1 RNA (Fig. 5C) confirmed that these products were located within the ICP4 gene. No hybridization was detected in lanes containing RT-PCR reactions with CHCC-OU2 or Md11p35 RNA (Fig. 5C). As a control for DNA contamination, no hybridization was detected when RNA templates were used for PCR without prior addition of Superscript II Reverse Transcriptase (data not shown). There was no hybridization of ICP4-specific probe to the 230-bp band in LAT-specific or ICP4-specific RT-PCR reactions with MSB-1 RNA(Fig. 5A, lane 6), or to the 175-bp band in Md11p35/CEF and MSB-1 (Fig. 5B, lanes 5 and 8), suggesting these bands represented nonspecific amplification products. Expression of MDV LATs in MDV OU2 cells combined with MDV protein expression patterns strongly suggest that MDV is in a latent state in these cells. Down regulation of MDV LATs in confluent MDV OU2 cells is associated with an increase in ICP4 gene expression as deduced from RT-PCR results (Fig. 5B, compare lanes 6 and 7). MDV ICP4 gene expression in subconfluent MDV OU2 cells was very low compared to expression in Md11p35-infected CEF cells and confluent MDV OU2 cells (Fig. 5B, lanes 5 and 6). These results are consistent with previous evidence that ICP4 transcripts are predominantly expressed in lytically infected cells and antisense transcripts are predominantly produced in latently infected cells (Cantello *et al.,* 1994; Li *et al.,* 1994).

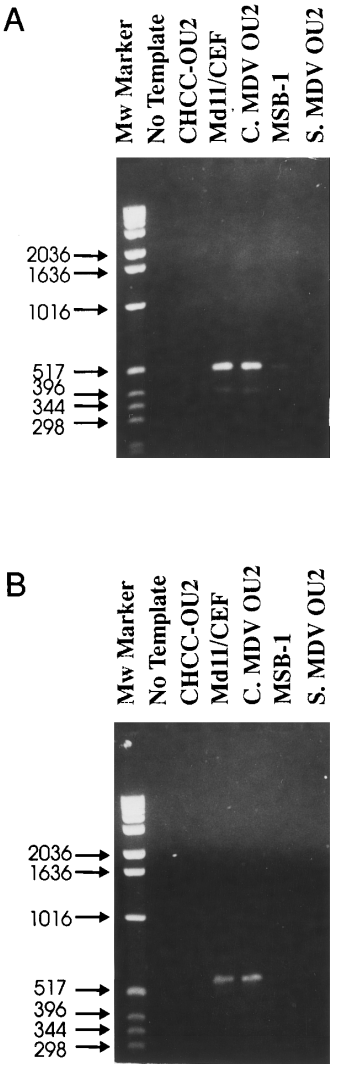

FIG. 4. RT-PCR amplification of a 530-bp fragment of gE and of a 560-bp fragment of gD transcripts. (A) Glycoprotein E-specific primers (E1 and E2) were used to amplify a 530-bp fragment using total RNA isolated from uninfected CHCC-OU2, Md11p35/CEF, confluent MDV OU2.2p33, MSB-1, and subconfluent MDV OU2.2p33 cells. (B) Glycoprotein D-specific primers were used to amplify a 560-bp fragment using total RNA isolated from uninfected CHCC-OU2, Md11p35/CEF, confluent MDV OU2.2p33, MSB-1, and subconfluent MDV OU2.2p33 cells. PCR-negative control was used in all reactions (No Template lane). A 1-kb DNA ladder marker (Life Technologies) was used for size comparison. Numbers at the left indicate sizes of MW marker fragments.

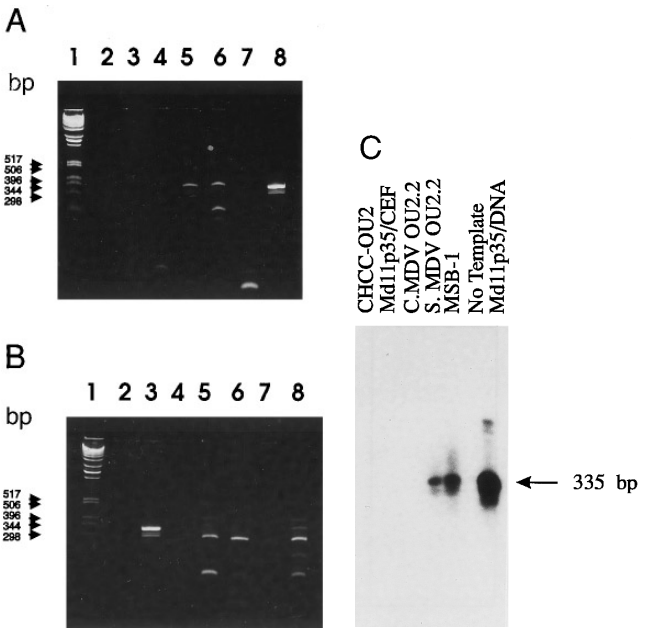

FIG. 5. RT-PCR amplification of a 335-bp fragment of MDV LATs and ICP4 gene. (A) LAT-specific primers were used to amplify a 335-bp fragment using total RNA isolated from CHCC-OU2 (lane 2), Md11p35/CEF (lane 3), confluent MDV OU2.2p31 (lane 4), subconfluent MDV OU2.2p31 (lane 5), MSB-1 cells (lane 6). Lanes 7 and 8 are PCR negative and positive control, respectively. (B) Primers specific for the MDV ICP4 transcript were used for RT-PCR amplification of a 300-bp fragment using RNA isolated from CHCC-OU2 (lane 4), Md11p35/CEF (lane 5), confluent MDV OU2.2p31(lane 6), subconfluent MDV OU2.2p31 (lane 7), MSB-1 cells (lane 8). Lanes 2 and 3 represent PCR negative and positive controls, respectively. A 1-kb DNA ladder marker (Life Technologies) (lane 1) was used for size comparison. Numbers at the left indicate approximate sizes of amplified fragments. Smaller size fragments are due to nonspecific amplification and were not detected using Southern blot analysis (data not shown). (C) Southern blot hybridization of LATs RT-PCR. Using ICP4 gene as a probe, RT-PCR products from MSB-1 and Subconfluent MDV OU2 cell strongly hybridized to a product of the expected size. No hybridization was detected in lanes containing uninfected OU2, confluent MDV OU2 cells, and Md11p35/CEF.

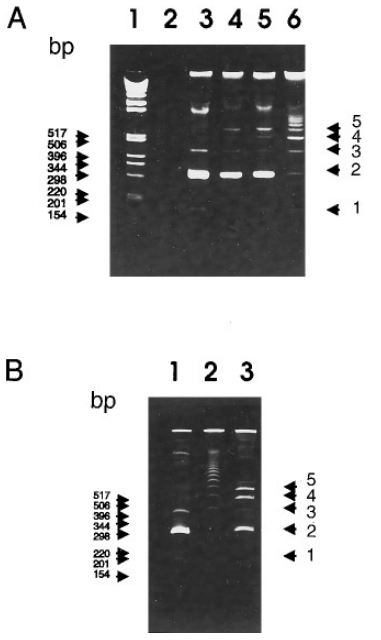

FIG. 6. PCR amplification of the 132-bp DR sequence. (A) DNA isolated from CHCC-OU2 (lane 2), Md11p15/CEF (lane 3), MDV OU2.2p12 (lane 4), MDV OU2.2p23 (lane 5), and Md11p86/CEF (lane 6) was used as template for PCR amplification. A 1-kb ladder marker (Life Technologies) (lane 1) was used for size comparison. DNA isolated from CHCC-OU2 cells (lane 2) served as negative control. (B) DNA isolated from Md11p15/CEF (lane 1), Md11p48/CEF (lane 2), and MDV OU2.2p28 (lane 3) was used as template for PCR amplification of DR sequences. Numbered arrows at the right indicate number of copies of the 132-bp DR sequence. Arrows at the left represent positions of selected bands from the 1-kb ladder marker (Life Technologies).

Serial *in vitro* passage does not cause attenuation of MDV genomes in MDV OU2 cells

A PCR assay developed by Silva (1992) was used to estimate the number of 132-bp DR sequences present in MDV genomes following serial *in vitro* passage of MDV OU2 cells and various control infections. Two hundred nanograms of total DNA from CHCC-OU2, Md11p15/CEF, Md11p28/CEF, Md11p48/CEF, Md11p86/CEF, MDV OU2.2p12, MDV OU2.2p23, and MDV OU2.2p28 cells was used as template for PCR amplification with a primer set flanking the 132-bp DR sequence (het region). In all reactions, except the CHCC-OU2 negative control, it was possible to detect a 185-bp amplified fragment corresponding to one copy of the 132-bp DR (Fig. 6A, lane 3). A 317-bp fragment corresponding to two copies of the 132-bp DR was predominant in reactions containing pathogenic Md11p15/CEF DNA as well as in DNA isolated from MDV OU2.2p12 and p23 (Fig. 6A, lanes 3, 4, and 5). Although the 317-bp fragment was present in DNA amplified from cells infected with Md11p86, the predominant PCR products were distributed over a range of bands representing between four and seven copies of the 132-bp DR sequence (Fig. 6A, lane 6).

Results of PCR analyses indicated that the 132-bp DR sequence in MDV DNA was not significantly expanded following extended serial *in vitro* passage in MDV OU2 cells. In contrast, and as expected, the 132-bp DR region was expanded following serial *in vitro* passage in secondary CEF cells. Therefore, similar to lymphoblastoid cell lines, MDV OU2 cells appear to stabilize MDV DNA. Despite many years in culture, lymphoblastoid cell lines are still capable of inducing MD in susceptible birds. Upon PCR analysis, 3–5 copies of the 132-bp DR sequences (data not shown) were detected in MDV genomes from MSB-1 cells. To further verify stabilization of MDV het region DNA in MDV OU2 cells, an equal passage variant of Md11 was generated in CEF cells. Due to a switch in passage numbering upon transfer to the CHCC-OU2 culture system, Md11p48/CEF represents an equal number of passages as MDV OU2.2p28 cells. A comparison of equal passage MDV in MDV OU2 cells (MDV OU2.2p28) and CEF cells (Md11p48) (Fig. 6B, lanes 3 and 2, respectively) clearly demonstrated that the 132-bp DR sequence is stabilized within MDV OU2 cells (Fig. 6B, lane 3) relative to an equal passage counterpart cultivated on CEF cells (Fig. 6B, lane 2). More than seven copies of the 132-bp DR were detected in PCR amplification (Fig. 6A, lane 6, and Fig. 6B, lane 2) of DNA from CEF cells infected with Md11p86 and Md11p48, respectively.

High passage MDV OU2 cells induce MD in susceptible chickens

Early passages of MDV OU2.2 cells can induce MD in susceptible birds within 3–5 weeks following intraperitoneal injection (Abujoub and Coussens, 1995). Stabilization of het region DNA in MDV OU2 cells suggested that, as with MDV lymphoblastoid cells, MDV pathogenicity should also be preserved in long term cultures of MDV OU2 cells. To confirm that the MDV genomes were not attenuated after extended serial *in vitro* culture in MDV OU2 cells, SPF chickens were inoculated with either MDV OU2.2p8 (low passage) or p23 (high passage) cells at 1 day of age. As a negative control, chickens were injected with parental CHCC-OU2 cells. Consistent with previous results, chickens injected with MDV OU2.2p8 cells developed classical signs of MD by the end of the fourth week postinoculation and either died or had to be euthanized by the end of the fifth week due to widespread paralysis. Necropsy revealed complete bursal atrophy and splenomegaly in all birds inoculated with MDV OU2.2p8. Chickens inoculated with MDV OU2.2 p23 also displayed signs of MD within 3–5 weeks postinoculation. In addition to severe paralysis, birds inoculated with MDV OU2.2p23 cells displayed severe weight loss when compared to CHCC-OU2- and MDV OU2.2p8-injected birds (data not shown). All birds in this group were euthanized by the end of the fifth week due to signs of severe illness. Upon necropsy, complete bursal atrophy and early signs of tumor formation were observed in

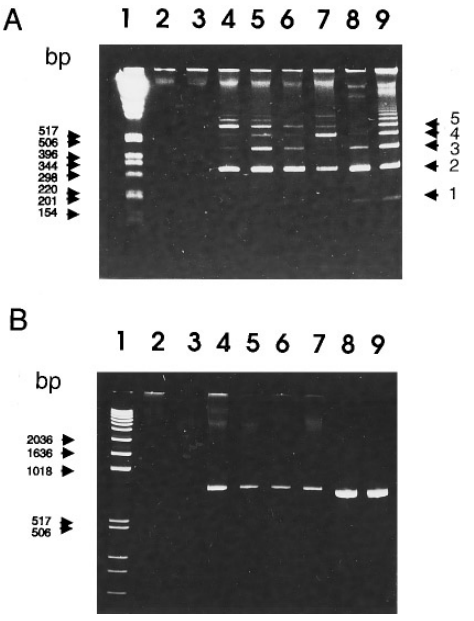

FIG. 7. PCR amplification of an 850-bp MDV pp38 gene. (A) DNA isolated from kidneys of birds inoculated with CHCC-OU2 (lanes 2 and 3), MDV OU2.2p8 (lanes 4 and 5), and MDV OU2.2p23 (lanes 6 and 7) was used as template for PCR amplification of the 132-bp DR region as described under Materials and Methods. DNA isolated from Md11p15 and p28/CEF (lanes 8 and 9) served as positive controls for DNA isolation and PCR reaction. (B) DNA isolated from kidneys of birds inoculated with CHCC-OU2 (lanes 2 and 3), MDV OU2.2p8 (lanes 4 and 5), and MDV OU2.2p23 (lanes 6 and 7) was used as template for PCR amplification of 850 bp as described under Materials and Methods. DNA isolated from Md11p15 and p28/CEF (lanes 8 and 9) served as positive controls for DNA isolation and PCR reaction. A 1-kb ladder marker (Life Technologies) (lane 1) was used for size comparisons. Sizes of selected fragments are indicated at the left.

livers of dissected birds. In contrast, chickens inoculated with parental CHCC-OU2 cells showed no clinical signs of MD. Control birds inoculated with CHCC-OU2 cells were euthanized at 16 weeks postinoculation. Necropsy revealed no signs of bursal atrophy, splenomegaly, or tumor formation.

To confirm that injected birds harbor MDV genomes, total cellular DNA isolated from kidneys was used as template for PCR amplification using a primer set specific for the 132-bp DR sequence (Silva, 1992) and for amplification of an 850-bp segment of the pp38 gene (Abujoub and Coussens, 1995). No PCR products were detected when DNA isolated from two separate control bird kidneys was used as template (Fig. 7A, lanes 2 and 3). In contrast, amplification products consistent with MDV sequences containing two to four copies of the 132-bp DR sequence were amplified from MDV OU2.2p8-inoculated bird kidneys (Fig. 7A, lanes 4 and 5). PCR reactions using DNA isolated from MDV OU2.2p23-injected birds resulted in amplification of products consistent with two to five copies of the 132-bp DR sequence (Fig. 7A, lanes 6 and 7). Md11p15/CEF DNA was used as a positive control for PCR reactions (Fig. 7A, lane 8). PCR reactions primed with a primer set specific for pp38 gene sequences amplified an 850-bp fragment in reactions containing DNA isolated from kidneys of birds inoculated with MDV OU2.2p8 (Fig. 7B, lanes 4 and 5) or MDV OU2.2p23 (Fig. 7B, lanes 6 and 7). No detectable MDV products were amplified in reactions containing DNA from kidneys of birds inoculated with CHCC-OU2 (Figs. 7A and 7B, lanes 2 and 3). The presence of MDV-specific PCR products indicated that both MDV OU2.2p8 and MDV OU2.2p23 cells were capable of transferring MDV to susceptible birds.

As an additional test for disseminated viremia in infected birds, blood was collected at the time of euthanization, and peripheral blood leukocytes (PBLs) were isolated from whole blood, washed with PBS, and cocultivated with secondary CEF cells. CEF monolayers cocultivated with PBLs from MDV OU2.2p8 and p23-injected birds displayed plaques consistent with MDV infection 3–5 days postcultivation. In contrast, and as expected, no plaques were observed on CEF monolayers cocultivated with PBLs from chickens injected with CHCC-OU2 cells. The disseminated viremia is an indication that MDV OU2 cells induced a systematic MDV infection in the inoculated birds and the virus was not localized to the site of inoculation (intraperitoneal cavity).

DISCUSSION

The MDV strain Md11-infected fibroblastic cell lines MDV OU2.1 and OU2.2 (Abujoub, and Coussens, 1995) are permissive for MDV replication. In contrast with primary CEF and DEF, these cell lines have an unlimited life span and have been in continuous culture for 2 years under conditions similar to that used for primary fibroblasts. Growth characteristics and appearance of MDV OU2 cells are indistinguishable from the parental CHCC-OU2-immortalized fibroblastic cell line (Ogura and Fujiwara, 1987). MDV OU2 cells display characteristics consistent with MDV genomes existing in a latent state, similar to that observed in MD-lymphoblastoid cell lines. However, the appearance of distinct plaques in confluent cell monolayers is more consistent with a lytic infection (Abujoub, and Coussens, 1995). Thus, MDV OU2 cells appear to "switch" from a latent to lytic infection, coincident with cells reaching confluence. Both MDV OU2 cell lines are capable of transferring MDV infection to CEF in culture and inducing clinical signs of MD in susceptible birds. Virus yields from these cell lines is comparable to or greater than yields produced by CEF cultures. Thus, CHCC-OU2- and MDV-infected derivatives provide an excellent system for cultivation of MDV vaccine viruses, production of MDV mutants (with coordinated expression of essential genes), and a model for herpesvirus latency/reactivation.

The overall aims of this study were to determine the status of MDV genomes in MDV OU2 cells and to exam-

TABLE 1

Summary of IIFA and RT-PCR Results

Cell type	Expressed gene					
	gB	gI	gD	gE	ICP4	LATs
MSB-1	ND	ND	−	±	+	++
Md11/CEF	++	++	++	++	++	−
Subconfluent MDV OU2	−	−	−	−	±	++
Confluent MDV OU2	++	++	++	++	++	±
CHCC-OU2	−	−	−	−	−	−

Note. ND, not determined; ±, low level of expression; +, normal level of expression; ++, high level of expression; −, not detected.

ine the effect of *in vitro* passage on stability of MDV genomes and virus pathogenesis within MDV OU2 cells. To achieve our first goal, we used IIFA staining and RT-PCR to examine differential gene expression in subconfluent versus confluent MDV OU2 cells. Our results, summarized in Table 1, support the hypothesis that MDV is in a latent state in MDV OU2 cells, similar to MDV lymphoblastoid cell lines. IIFA staining and RT-PCR data clearly demonstrate that expression of structural glycoproteins (glycoproteins B, I, D, and E) which are encoded by late genes and are mainly expressed in lytically infected cells, occurs only in confluent cell monolayers. This pattern of protein expression is consistent with MDV genomes existing in a latent state in subconfluent MDV OU2 cells. MDV appears to be reactivated and a full cycle of lytic infection initiated after cells reach confluence.

Cantello *et al.* (1994) and Li *et al.* (1994) separately reported the identification of transcripts (MDV LATs) that map antisense to the ICP4 homolog gene of MDV. MDV LATs are expressed at a significantly higher level in lymphoblastoid cell lines (MSB-1 and RPL1) than in lytically infected cells. Based on these findings, it was speculated that MDV LATs may play a role in maintenance of latency by negatively regulating MDV ICP4 expression (Cantello *et al.,* 1994; Li *et al.,* 1994). Using RT-PCR, we examined the level of expression of MDV LATs in MDV OU2 cells from subconfluent cultures versus confluent cultures. Expression of MDV LATs in subconfluent MDV OU2 cells was comparable to that observed in MSB-1 cells. MDV LAT expression is down regulated when cells become confluent and was not detected or detected at a very low levels when cells were allowed to display plaques characteristic of lytic MDV infection. When the level of MDV ICP4 expression was examined, the level of ICP4 expression was inversely proportional to the level of MDV LAT expression. The similarity between MDV LAT/ICP4 expression patterns in subconfluent MDV OU2 cells to MSB-1 cells strongly supports our hypothesis that MDV exists in a latent state in MDV OU2 cells in subconfluent cultures. In addition, our results confirm the hypothesis of Cantello *et al.* (1994) and Li *et al.* (1994) that MDV LATs may serve to down regulate expression of MDV ICP4.

The factors responsible for the switch from a latent state in subconfluent cultures to a lytic infection in confluent monolayers is not understood. Generally, factors important in determining latent or lytic infection cycles in many herpes viruses are cellular activators or repressors which in turn activate or repress viral gene products responsible for determining the pathway of viral infection (Garcia-Blanco and Cullen, 1991). MDV OU2 cells are similar to their parental CHCC-OU2 cells in being contact inhibited. It is possible that changes in cell growth associated with confluence and contact inhibition act to trigger reactivation of MDV leading to expression of the full complement of MDV genes and lytic replication. It is tempting to speculate that such a trigger may be a regulator (repressor) of MDV LAT expression. As in CEF cells, lytic replication of MDV in confluent MDV OU2 cell cultures appears to be of the productive restrictive class with no infectious virions released into the culture fluid (Abujoub and Coussens, unpublished observations)

Serial *in vitro* passage of virulent MDV strains on CEF cells results in attenuation and loss of oncogenicity which is correlated with het region expansion. In this report, we provide evidence that a highly variable MDV genome region is stabilized in MDV OU2 cells, and after over 2 years of continuous *in vitro* culture MDV from MDV OU2 cells is still oncogenic. PCR analyses showed that the number of 132-bp DR sequences did not change significantly in MDV genomes after more than 30 *in vitro* passages. Whereas, Md11 passed in CEF culture for the same number of passages (Md11p48/CEF) exhibited significant expansion of the 132-bp DR region. The number of 132-bp DR sequences in oncogenic MDV strains is typically between 2 and 5 copies, whereas the average number in strains attenuated by *in vitro* cultivation on CEF cells is three to greater than seven copies. These results suggest that, as with MDV lymphoblastoid cell lines MDV OU2 cell lines tend to stabilize the MDV genome. However, CHCC-OU2 cells are nontumorigenic and thus may offer an attractive alternative for production of recombinant MDV which may be unstable in CEF cultures.

Marek's disease can be induced by injection of MDV-infected cells into susceptible birds. Tumor incidence can reach 100%, but is dependent on various natural and experimental conditions and factors, for example, virus strain and dose, site of injection, age at primary exposure, and the genetic background of the birds (Calnek and Witter, 1991). Oncogenic serotype 1 MDV-infected CEF, DEF, and CKC cells as well as some producer MD-lymphoblastoid cell lines such as MSB-1, CU36, and CU41 are able to induce MD in susceptible birds. Similarly, MDV OU2 cells are capable of inducing MD in susceptible birds. With serial *in vitro* passage, in CEF, DEF, and CKC cells oncogenic MDV strains are rapidly

attenuated. Nononcogenic MDV strains tend to lose potency as vaccines with continuous *in vitro* cultivation. Propagation in lymphoblastoid cells can stabilize viral genomes and preserve the initial character of resident MDV strains. However, Lymphoblastoid cells are oncogenic and have not been found tp harbor common vaccine strains of MDV.

Since, PCR analyses showed that the 132-bp DR region of MDV was stabilized during prolonged cultivation in MDV OU2 cells. It was important to determine if prolonged cultivation of MDV in CHCC-OU2 cells could preserve oncogenicity. Susceptible birds inoculated with MDV OU2.2p23 developed signs of MD, including severe weight loss and paralysis similar to those inoculated with MDV OU2.2p8. PCR amplification of DNA isolated from kidneys of infected birds demonstrated systemic presence of MDV and indicated that infectious virions contained between two and five copies of the MDV 132-bp DR sequences. Our PCR and *in vivo* data indicates that MDV genomes are stabilized within MDV OU2 cell lines, similar to that seen in lymphoblastoid cell lines. However, unlike lymphoblastoid cell lines, the parental CHCC-OU2 cells are nononcogenic (Abujoub and Coussens, 1995; Ogura and Fujiwara, 1987). No evidence of tumor formation or any illness in birds inoculated with CHCC-OU2 parental cells up to 16 weeks postinoculation has been found.

This report shows, for the first time, the presence of latent MDV genomes in a sustainable fibroblast cell line. Establishment and characterization of these cell lines will reduce many of the difficulties associated with MDV experimentation and vaccine production. For example, studying differential gene expression between subconfluent and confluent MDV OU2 cells will help in the search for key switches in MDV latency. A key difference noted in the present report between latent and lytic MDV OU2 infections is down regulation of MDV LAT expression coincident with culture confluence. Regulation of these transcripts in subconfluent and confluent MDV OU2 cells is currently under investigation. Stabilization of MDV genomes within MDV OU2 cell lines will make this system ideal for generation of mutants and recombinant viruses. In addition, propagation of MDV vaccine strains on CHCC-OU2 cells, unlike on CEF, offers the possibility that continuous passage will not offset efficacy of MDV vaccines.

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